LONG-TERM MYOCARDIAL PRESERVATION: EFFECTS OF HYPERKALEMIA, SODIUM CHANNEL, AND $NA^+/K^+/2CL^-$ COTRANSPORT INHIBITION ON EXTRACELLULAR POTASSIUM ACCUMULATION DURING HYPOTHERMIC STORAGE

Andrew K. Snabaitis, PhD[a]
Michael J. Shattock, PhD[b]
David J. Chambers, PhD[a]

Objectives: **We previously demonstrated improved myocardial preservation with polarized (tetrodotoxin-induced), compared with depolarized (hyperkalemia-induced), arrest and hypothermic storage. This study was undertaken to determine whether polarized arrest reduced ionic imbalance during ischemic storage and whether this was influenced by $Na^+/K^+/2Cl^-$ cotransport inhibition.** ***Methods:*** **We used the isolated crystalloid perfused working rat heart preparation (1) to measure extracellular K^+ accumulation (using a K^+-sensitive intramyocardial electrode) during ischemic (control), depolarized (K^+ 16 mmol/L), and polarized (tetrodotoxin, 22 μmol/L) arrest and hypothermic (7.5°C) storage (5 hours), (2) to determine dose-dependent (0.1, 1.0, 10 and 100 μmol/L) effects of the $Na^+/K^+/2Cl^-$ cotransport inhibitor, furosemide, on extracellular K^+ accumulation during polarized arrest and 7.5°C storage, and (3) to correlate extracellular K^+ accumulation to postischemic recovery of cardiac function.** ***Results:*** **Characteristic triphasic profiles of extracellular K^+ accumulation were observed in control and depolarized arrested hearts; a significantly attenuated profile with polarized arrested hearts demonstrated reduced extracellular K^+ accumulation, correlating with higher postischemic function (recovery of aortic flow was 54% ± 4% [*P* = .01] compared with 39% ± 3% and 32% ± 3% in depolarized and control hearts, respectively). Furosemide (0.1, 1.0, 10, and 100 μmol/L) modified extracellular K^+ accumulation by –18%, –38%, –0.2%, and +9%, respectively, after 30 minutes and by –4%, –27%, +31%, and +42%, respectively, after 5 hours of polarized storage. Recovery of aortic flow was 53% ± 4% (polarized arrest alone), 56% ± 8%, 70% ± 2% (*P* = .04 vs control), 69% ± 4% (*P* = .04 vs control), and 65% ± 3% (*P* = .04 vs control), respectively.** ***Conclusions:*** **Polarized arrest was associated with a reduced ionic imbalance (demonstrated by reduced extracellular K^+ accumulation) and improved recovery of cardiac function. Further attenuation of extracellular K^+ accumulation (by furosemide) resulted in additional recovery. (J Thorac Cardiovasc Surg 1999;118:123-34)**

Within seconds of the onset of an *unprotected* period of ischemia, extracellular potassium (K^+_e) begins to accumulate in a characteristic triphasic manner and is associated with a concomitant depolarization of the myocardial resting membrane potential (E_m).[1] The extracellular potassium concentration is an important determinant of the E_m, and this relationship is exploited by hyperkalemic preservation solutions, used

From Cardiac Surgical Research[a] and Cardiovascular Research,[b] The Rayne Institute, St Thomas' Hospital, London, United Kingdom.

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Address for reprints: D. J. Chambers, PhD, Cardiac Surgical Research/Cardiothoracic Surgery, The Rayne Institute, St Thomas' Hospital, London, SE1 7EH, United Kingdom.

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Table I. *Composition of solutions used to compare K^+_e accumulation during depolarized and polarized long-term hypothermic preservation and subsequent postischemic recovery of cardiac function*

Constituent (mmol/L)	*Control (KH buffer)*	*Depolarized (high K^+)*	*Polarized (TTX)*
Na^+	144	144	144
K^+	5.9	16.0	5.9
Cl^-	127	142	127
Mg^{2+}	1.2	1.2	1.2
Ca^{2+}	1.4	1.4	1.4
HCO_3^-	25	25	25
$H_2PO_4^-$	1.2	1.2	1.2
SO_4^{2-}	1.2	1.2	1.2
Glucose	11.1	11.1	11.1
TTX (μmol/L)	—	—	22
pH at 7.5°C	8.3	8.2	8.3

KH, Krebs-Henseleit; *TTX,* tetrodotoxin.

for cardioplegic arrest and long-term hypothermic storage of the heart during clinical transplantation. Infusion of these solutions induces a rapid K^+-induced depolarization of the E_m and a flaccid diastolic arrest. However, hyperkalemia-induced depolarization of the resting E_m may predispose the myocardium to the accumulation of intracellular sodium (Na^+_i)[2] via activation of the sodium "window current" (I_{Naw}). We[3] have previously shown that, during 5 hours of global hypothermic (7.5°C) ischemic storage, a solution containing a 16-mmol/L concentration of K^+ depolarizes and maintains the E_m of hearts at approximately –50 mV; inasmuch as this is within the required voltage range (–65 to –15 mV) for activation of I_{Naw}, it is likely that I_{Naw} would become activated, supporting the evidence that this current may be involved in the pathogenesis of ischemia and reperfusion injury.[4] Hyperkalemia may also predispose the myocardium to calcium accumulation via activation of a voltage-dependent calcium "window current" (I_{Caw}),[5] and this is supported in studies by Lopéz and colleagues,[6] who have demonstrated that isolated myocytes exposed to a 16-mmol/L concentration of K^+ rapidly accumulate calcium.

In contrast, preservation solutions that are formulated to maintain the myocardial E_m close to the resting level of approximately –80 mV in a more polarized state[3] may prevent the activation of I_{Naw} and I_{Caw}. At this voltage, Ca^{2+} and Na^+ channels should be in a closed state and the driving force for potassium ions greatly reduced as the E_m is close to the reversal potential for potassium (E_K). Therefore, a polarized E_m should lead to an attenuation of ionic imbalance, including Na^+_i,[7] Ca^{2+}_i,[6] and K^+_e accumulation, and thereby reduce metabolic demand[8]; this should prove to be more beneficial to the ischemic myocardium.[3]

Profound *hypothermia* during ischemia has been shown to be associated with large increases in Na^+_i.[9] The $Na^+/K^+/2Cl^-$ cotransporter has been implicated as one of several possible routes involved in the observed sodium overload.[10] Furosemide, a loop diuretic that has been shown to inhibit this cotransport pathway, may prove to be of benefit to the postischemic myocardium[11]; however, inhibition may also cause a concomitant elevation of K^+_e, resulting from the unidirectional nature of the $Na^+/K^+/2Cl^-$ cotransporter.[12] The effects of $Na^+/K^+/2Cl^-$ cotransport inhibition on K^+_e accumulation during *hypothermic* ischemic storage are largely unknown.

Therefore, the objectives of this study were (1) to investigate K^+_e accumulation during ischemic, high K^+–induced (depolarized) and tetrodotoxin-induced (polarized) arrest and *hypothermic* storage, (2) to determine the dose-dependent effects of $Na^+/K^+/2Cl^-$ cotransport inhibition on K^+_e accumulation during tetrodotoxin-induced (polarized) arrest and *hypothermic* storage, and (3) to correlate any changes in K^+_e accumulation to the recovery of postischemic cardiac function.

Materials and methods

Animals. Hearts were obtained from male Wistar rats (Bantin and Kingman Ltd, Hull, United Kingdom) weighing 200 to 250 g. All animals received humane care in accordance with the "Guidance on the Operation of the Animals (Scientific Procedures) Act 1986," published by Her Majesty's Stationery Office, London, England.

Experimental preparation. The isolated perfused working rat heart preparation was used for this study. In brief, it is a left-sided heart preparation in which oxygenated Krebs-Henseleit perfusion buffer (at 37°C) enters the cannulated left atrium at a pressure equivalent to 20 cm H_2O. The perfusate passes to the left ventricle, from which it is spontaneously ejected through an aortic cannula against a hydrostatic pressure equivalent to 100 cm H_2O. Exclusion criteria imposed in this study rejected hearts that produced an aortic flow less than 50 mL/min or a coronary flow in excess of 26 mL/min after 20 minutes of the control (preischemic) working period. Coronary flow from the right side of the heart can be sampled for enzyme analysis or pooled and recirculated with the aortic outflow. The Krebs-Henseleit bicarbonate perfusion buffer contained (in millimoles per liter) NaCl 119, $NaHCO_3$ 25, KCl 4.75, $MgSO_4$ 1.2, KH_2PO_4 1.18, $CaCl_2$ 1.4, and glucose 11.1 at a pH of 7.4 when gassed with 95% oxygen and 5% carbon dioxide at 37°C (see Table I for detailed composition). This solution was filtered through a 5-μm porosity cellulose nitrate filter before use and was continually passed through an in-line 5-μm porosity filter during the working period of the study. The filter was changed before the reperfusion working period.

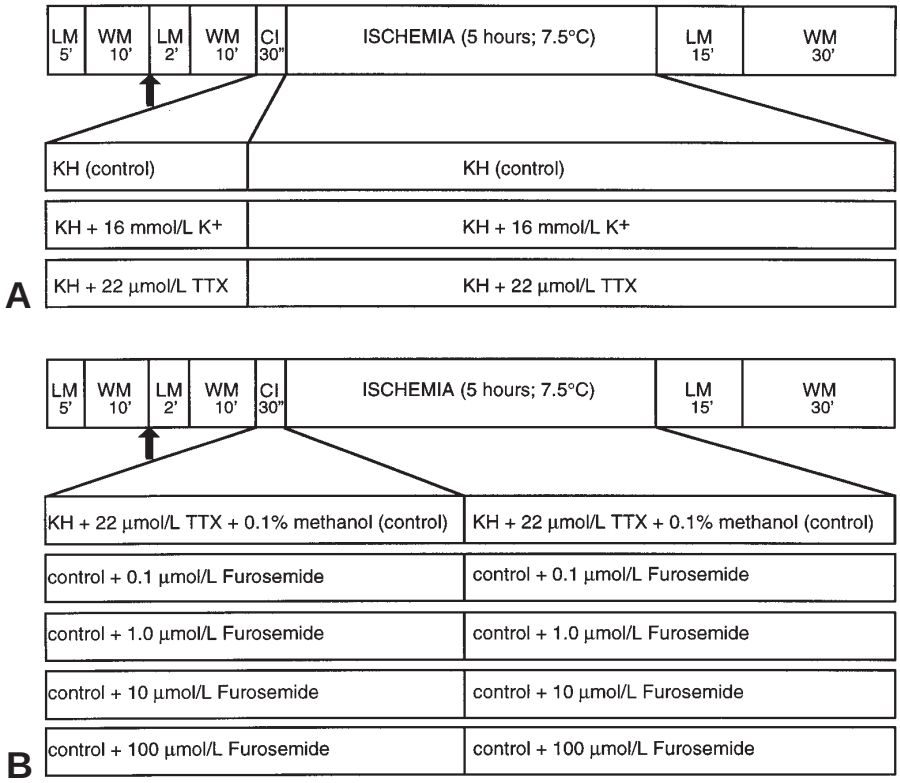

Fig 1. The experimental protocols for study A and study B are represented diagrammatically. The *arrows* indicate the stage during the protocol at which the electrodes were inserted into the myocardium. *LM*, Langendorff mode; *WM*, working mode; *CI*, cardioplegic infusion; *KH*, Krebs-Henseleit; *TTX*, tetrodotoxin.

During the hypothermic storage period, each heart was maintained in a sealed temperature-controlled heart chamber at 7.5°C.

Perfusion protocol. After cannulation of the aorta, the heart was perfused with Krebs-Henseleit buffer in the Langendorff mode for a 5-minute stabilization period. During this stabilization period, the pulmonary artery was cut and the pulmonary veins to the left atrium were cannulated. The heart was then converted to working mode perfusion, and preischemic (control) cardiac function (aortic flow, aortic pressure, heart rate, coronary flow, and cardiac output) was determined during two 10-minute periods of working mode perfusion; this was interspersed with a 5-minute period of Langendorff mode perfusion (Fig 1, *A* and *B*) when the K^+-sensitive electrode (Fig 2) was stitched into the myocardium.

Two studies were conducted:

Study A: Depolarized versus polarized storage. To compare the effects of ischemic arrest, high K^+ (depolarized) arrest, and tetrodotoxin-induced (polarized) arrest and storage on K^+_e accumulation, hearts were infused (over 30 seconds) with 2 mL of either Krebs-Henseleit (control) buffer, Krebs-Henseleit buffer + 16 mmol/L K^+, or Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin via a self-sealing multi-injection port at 21°C (Fig 1, *A*). This volume and rate of delivery had previously been determined as the optimum in terms of functional recovery in the rat heart. Individual hearts were then stored for 5 hours in separate chambers containing 9 mL of the arresting solution (Krebs-Henseleit buffer, Krebs-Henseleit buffer + 16 mmol/L K^+, or Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin) so that the heart (with electrodes in place) was totally immersed in the hypothermic (7.5°C) storage solution for 5 hours (see Table I for arrest/storage solution composition).

Study B: Inhibition of $Na^+/K^+/2Cl^-$ cotransport during polarized storage. Furosemide (Sigma-Aldrich, Dorset, United Kingdom), a $Na^+/K^+/2Cl^-$ cotransport inhibitor, was dissolved in methanol, and different concentrations of stock solutions were kept at 4°C for 5 days; the final methanol concentration in the storage solution was 0.1% in all groups of study B. To investigate the effects of the $Na^+/K^+/2Cl^-$ cotransport inhibitor furosemide on K^+_e accumulation during polarized arrest and storage, hearts were infused (over 30 seconds) with 2 mL of Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin + 0.1% methanol (control) or control + 0.1, 1.0, 10, or 100 μmol/L furosemide via a self-sealing multi-injection port at 21°C (see Fig 1, *B*). Individual hearts were then stored for 5 hours in separate chambers containing 9 mL of the arresting solution (Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin +

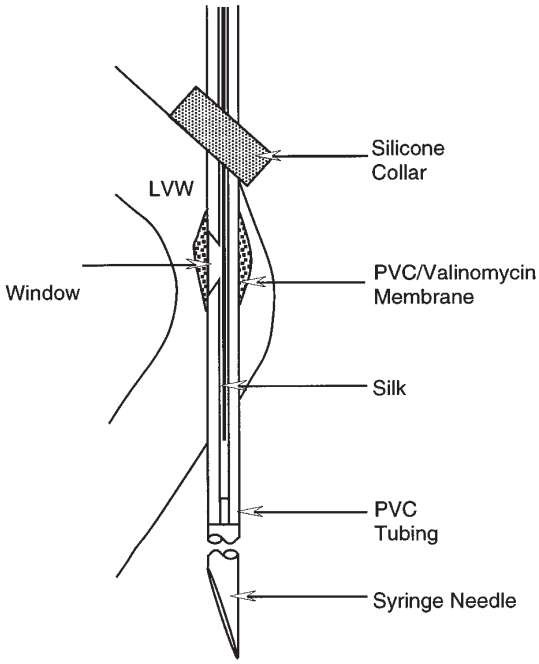

Fig 2. Diagrammatic representation of a cross section through a "side-window" PVC/valinomycin electrode, showing the electrode stitched into the left ventricular wall *(LVW)* for on-line determination of myocardial K^+_e during long-term preservation.

0.1% methanol [control] or control + 0.1, 1.0, 10, or 100 μmol/L furosemide) so that the heart (with electrodes in place) was totally immersed in the hypothermic (7.5°C) storage solution for 5 hours (see Table II for arrest/storage solution composition). K^+_e was continuously monitored throughout the storage period and the signal was captured by using a purpose-built electrode amplifier and passed through a 1.0-Hz low-pass filter, displayed on a digital storage oscilloscope (Gould model 1425, Gould, Inc, Oxnard, Calif) and recorded on a chart recorder (Gould model RS 3200).

Postischemic analysis. After storage, all hearts in study A and 8 hearts per group in study B were reperfused with Krebs-Henseleit buffer, initially in the Langendorff mode for 15 minutes and then in the working mode for a further 30 minutes. During working mode reperfusion, recovery of postischemic cardiac function was determined and expressed as a percentage of the averaged cardiac function measured before and after electrode insertion, during preischemic working mode perfusion. In study B, hearts (n = 4) that were used for K^+_e measurement were freeze-clamped at the end of the storage period for analysis of tissue water content.

Potassium mini-electrode preparation. Potassium sensitive "end-window" mini-electrodes, incorporating a polyvinyl chloride (PVC)/valinomycin membrane, were first used by Hill and coworkers[13] for the on-line measurement of myocardial and intravascular potassium concentration. In this study, this "end-window" electrode configuration was replaced by an alternative "side-window" configuration, thereby improving the electrode sensor for measurement of intramyocardial potassium. Our side-window configuration (Fig 2) allowed the electrodes to be stitched into the myocardium, eliminating the possibility of the electrode becoming dislodged by movement of the heart during aerobic perfusion and reperfusion. These electrodes were used to measure intramyocardial K^+_e accumulation as an index of ionic imbalance during (1) unprotected ischemia, high K^+, and tetrodotoxin-induced arrest and storage and (2) tetrodotoxin-induced polarized arrest combined with $Na^+/K^+/2Cl^-$ cotransport inhibition, in hearts subjected to long-term preservation.

Table II. *Composition of solutions used to study the dose-dependent effect of furosemide on K^+_e accumulation during polarized arrest and storage and subsequent postischemic recovery of cardiac function*

Constituent	*Control*	*Group I*	*Group II*	*Group II*	*Group IV*
Na^+ (mmol/L)	144	144	144	144	144
K^+ (mmol/L)	5.9	5.9	5.9	5.9	5.9
Cl^- (mmol/L)	127	127	127	127	127
Mg^{2+} (mmol/L)	1.2	1.2	1.2	1.2	1.2
Ca^{2+} (mmol/L)	1.4	1.4	1.4	1.4	1.4
HCO_3^- (mmol/L)	25	25	25	25	25
$H_2PO_4^-$ (mmol/L)	1.2	1.2	1.2	1.2	1.2
SO_4^{2-} (mmol/L)	1.2	1.2	1.2	1.2	1.2
Glucose (mmol/L)	11.1	11.1	11.1	11.1	11.1
TTX μmol/L	22	22	22	22	22
Methanol (%)	0.1	0.1	0.1	0.1	0.1
Furosemide (μmol/L)	—	0.1	1.0	10	100
pH at 7.5°C	8.3	8.3	8.3	8.3	8.3

KH, Krebs-Henseleit; *TTX*, tetrodotoxin.

Preparation of PVC/valinomycin ion–selective membrane for the "side-window" electrode. The electrode matrix consisted of a PVC/valinomycin mixture containing 2.0 mg valinomycin, 0.5 mg potassium tetrakis (4-chlorophenyl borate), 150 mg *bis*(2-ethylhexyl) sebacate, and 66.0 mg PVC (molecular weight 200,000) dissolved in 4 mL of tetrahydrofuran. All chemicals were obtained from Fluka Chemicals (Sigma-Aldrich Corporation, St Louis, Mo). The mixture was stirred for 2 hours at room temperature and then allowed to stand unsealed for 48 hours at room temperature to allow the tetrahydrofuran to evaporate.

Electrodes for the measurement of K^+_e were prepared from PVC tubing with a 1.0 mm inner diameter × 1.6 mm outer diameter (Portex Ltd, United Kingdom) pulled to a taper over a Bunsen flame. The lumen of the PVC tube was then examined under a low-power microscope (×30 magnification) and, if wide enough (>0.25 mm) for a piece of silk to be inserted down the lumen, the PVC tube was trimmed to a length of approximately 10 cm for the next stage. A small amount of

epoxy resin was sucked into the tapered end to a distance of 5 mm and allowed to dry. The tapered end of the PVC tube was bent back and a small portion of the side wall of the tubing was cut away with a scalpel blade to leave a "window" in the side of the electrode. Through this window a piece of 8-0 or 6-0 silk (Mersilk, Ethicon Ltd) was passed up toward the wider end of the PVC tube. A small (1 mm) collar of silicone rubber tubing was positioned behind the window on the PVC tubing to act as a stop during electrode insertion (Fig 2).

The electrode was filled with a solution of KCl 4.0, NaCl 146, $CaCl_2$ 1.4, and $MgCl_2$ 1.2 (in millimoles per liter). The PVC matrix was then redissolved in 750 μL of tetrahydrofuran and, using a clean glass rod, a small amount of the PVC/valinomycin matrix was coated over the side window to form a membrane. Two coats of the PVC/valinomycin matrix were applied to obtain a complete and robust covering of the window. The electrodes were prepared 24 hours before use and stored in a solution identical to the electrode filling solution. A syringe needle was attached to the end of the electrode with cyanoacrylate adhesive.

Calibration of electrodes. Pre-experiment calibration of the potassium electrodes was performed at both 7.5°C and 37°C in solutions containing potassium concentrations (in millimoles per liter) of 4, 5.93 (the same as Krebs-Henseleit buffer), 16, and 32 (where total $K^+ + Na^+ = 150$ mmol/L), $CaCl_2$ 1.2, and $MgCl_2$ 1.2; electrodes responded in a Nernstian manner at both 7.5°C (54.8 ± 0.3 mV/decade) and 37°C (60.5 ± 0.3 mV/decade). At the end of an experimental protocol, the potassium electrodes were recalibrated in the same solutions at both 7.5°C (55.6 ± 0.4 mV/decade) and 37°C (60.6 ± 0.3 mV/decade) to ensure that the electrodes were undamaged during the experiment and functioning satisfactorily; experiments were discarded if the difference between the pre-experiment and post-experiment calibration at 37°C was more than 1 mV. To test whether electrodes behaved similarly during pre-experiment calibration and when stitched into the heart, in vitro calibration of the electrodes was performed by perfusing the heart with Krebs-Henseleit–based solutions at 37°C (59.5 ± 0.8 mV/decade) containing KCl at concentrations of 4, 5.93 (the same as Krebs-Henseleit solution), 16, and 32 mmol/L at the end of the protocol. The selectivity of the potassium electrodes over sodium ($K_{K\text{-}Napot}$) at 7.5°C and 37°C was determined to be 0.0019 and 0.0034, respectively, by measuring the voltage difference (V_{diff}) induced when increasing the concentration of the principal interfering ion (Na^+) from 140 mmol/L to 170 mmol/L while keeping the K^+ concentration constant at 0.4 mmol/L. The time taken for electrodes to respond and achieve steady state after immersion in a 4 mmol/L K^+ calibration solution was 0.90 ± 0.04 second at 7.5°C and 0.86 ± 0.04 second at 37°C.

Myocardial water content. In those hearts that were freeze-clamped at the end of storage (study B), percentage water content of ventricular biopsy specimens was calculated by the relative change in biopsy weight after drying in an oven for 24 hours at 80°C.

Statistical analysis. Hearts were randomized into appropriate groups before experiments by means of a computerized randomization procedure. In study A, there were 6 hearts per group; in study B, 12 hearts per group were used to determine (1) the effects of furosemide on K^+_e accumulation (4 hearts per group) and (2) the recovery of cardiac function (8 hearts per group) after tetrodotoxin-induced (polarized) arrest. All data are presented as mean ± standard error of the mean.

Preischemic cardiac function, measured during two periods of 10-minute working mode perfusion (see Fig 1), were compared by a paired 1-tailed Student *t* test to determine the effect of electrode insertion. To determine differences in recovery of function between groups, data were subjected to analyses by a 1-way analysis of variance and, if statistical significance was achieved, Dunnett's (for group vs control comparison) and Tukey's (for group vs group comparison) modified *t* tests (to account for multiple comparisons) were used. Differences were considered significant at the 95% confidence limit. All statistical analysis was conducted on a Macintosh microcomputer using Statview SE + Graphics (version 1.03, Abacus Concepts Inc, Berkeley, Calif).

Results

Study A

K^+_e accumulation during ischemic arrest, high K^+ arrest, and tetrodotoxin arrest and storage. The relationship between K^+_e accumulation and ischemic duration in control hearts (ischemic arrest) and hearts arrested with either 16-mmol/L K^+ (high K^+) or 22-μmol/L tetrodotoxin is shown in Fig 3, *A.* Baseline K^+_e was 5.93 mmol/L (ie, the potassium concentration in Krebs-Henseleit buffer perfusate before the onset of ischemia). From the onset of ischemia, K^+_e accumulated during hypothermic storage in a typical triphasic profile in all groups; in the control group of hearts, K^+_e increased to an initial (phase 1) peak of 11.2 ± 0.7 mmol/L after 15 minutes and decreased to 9.6 ± 0.6 mmol/L after 75 minutes (during phase 2) before increasing a second time to a peak of 20.5 ± 2.7 mmol/L (during phase 3) immediately before reperfusion. Infusion of hearts for 30 seconds with Krebs-Henseleit buffer containing high K^+ (at a final concentration of 16 mmol/L) rapidly increased the K^+_e to 16.3 ± 0.4 mmol/L (Fig 3, *A*). During ischemia, K^+_e increased further to an initial peak of 18.8 ± 4.8 mmol/L at 20 minutes during phase 1, then decreased during phase 2 to 16.6 ± 2.0 mmol/L after 90 minutes, before again increasing to 32.2 ± 3.6 mmol/L during phase 3, before reperfusion. In tetrodotoxin-treated hearts, peak K^+_e accumulation during phase 1 was delayed to 45 minutes and had risen to only 8.8 ± 0.4 mmol/L. There was a slight decrease to 8.4 ± 1.9 mmol/L after 105 minutes during phase 2 before K^+_e accumulation increased to 14.1 ± 1.7 mmol/L during phase 3, before reperfusion (Fig 3, *A*).

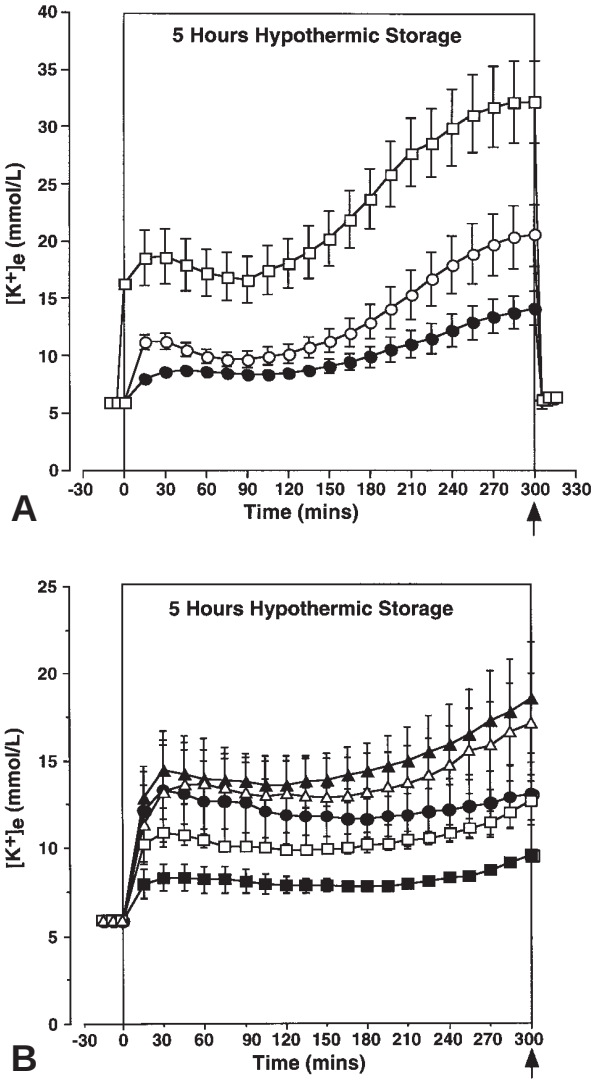

Fig 3. All hearts were initially perfused in the working mode for 10 minutes before cardioplegic arrest and hypothermic (7.5°C) storage for 5 hours. Changes in myocardial K^+_e (mmol/L) were then determined with potassium-sensitive "side-window" flexible electrodes. **A,** Hearts were arrested and stored with Krebs-Henseleit buffer alone (control: *open circles*), high K^+ *(open squares)*, or tetrodotoxin *(filled circles)*. *Arrow* indicates onset of reperfusion; n = 6 hearts per group. **B,** Hearts were arrested and stored with Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin + 0.1% methanol (control; *filled circles*), control + 0.1 μmol/L *(open squares)*, 1.0 μmol/L *(filled squares)*, 10 μmol/L *(open triangles)*, and 100 μmol/L *(filled triangles)* furosemide; n = 4 hearts per group. *Arrow* indicates time of freeze-clamping of hearts for tissue weight determination. All values represent the mean ± standard error of mean; baseline K^+_e during preischemic aerobic perfusion and postischemic reperfusion was equal to that of the Krebs-Henseleit buffer perfusate (5.93 mmol/L).

Rate of K^+_e accumulation during high K^+– and tetrodotoxin-induced arrest and storage. The rate of change in K^+_e accumulation during ischemic storage is shown in Fig 4, *A*; the most rapid rate of increase in K^+_e in the control group (0.9 ± 0.3 mmol/L per minute) occurred during the second minute of ischemia. In contrast, the most rapid rate of K^+_e accumulation in the high K^+–treated and tetrodotoxin-treated groups was 0.5 ± 0.3 and 0.3 ± 0.1 mmol/L per minute, respectively, and occurred during the fourth minute after the onset of ischemia.

Recovery of function after ischemic arrest, high K^+ arrest, or tetrodotoxin arrest and storage. Preischemic cardiac function did not differ before and after electrode insertion in each group (see Fig 1); consequently, the mean of the combined data for each heart over the 20-minute working mode perfusion period was used to determine percentage recovery and subsequent mean values (Table III). Aortic flow recovered to 54% ± 4% in the tetrodotoxin-treated hearts, and this was significantly ($P = 0.01$) higher than in the control (32% ± 4%) and the high K^+ (39% ± 3%) groups of hearts (Fig 5, *A*). Other indices of cardiac function (aortic pressure, heart rate, coronary flow, cardiac output, stroke volume, and stroke work) were also measured for all groups, and the recovery of these indices and for aortic flow is shown in Table III.

Study B

Effect of furosemide on K^+_e accumulation during polarized arrest and storage. The profiles of myocardial K^+_e accumulation in the control solution (Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin) and addition of 0.1, 1.0, 10, or 100 μmol/L furosemide to the control solution are shown in Fig 3, *B*. After the onset of ischemia in the control group (Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin + drug vehicle [0.1% methanol]), K^+_e during phase 1 increased to a peak of 13.3 ± 2.6 mmol/L after 30 minutes. Myocardial K^+_e then decreased to 11.7 ± 1.7 mmol/L during phase 2 before increasing to 13.1 ± 1.8 mmol/L at the end of storage during phase 3. In hearts treated with 0.1, 1.0, 10, and 100 μmol/L furosemide, peak K^+_e accumulation during phase 1 was 10.9 ± 1.0, 8.4 ± 0.8, 13.7 ± 2.6, and 14.5 ± 2.2 mmol/L, respectively. During phase 2, K^+_e decreased to 9.8 ± 0.2, 7.8 ± 0.4, 12.9 ± 2.3, and 13.6 ± 1.4 mmol/L before increasing to 12.7 ± 1.1, 9.6 ± 0.2, 17.1 ± 2.9, and 18.6 ± 3.2 mmol/L, respectively, at the end of phase 3. Furosemide (0.1 and 1.0 μmol/L) reduced the total K^+_e accumulation (area under curve) by 28% and 65% ($P = .02$ vs control group for both), whereas higher concentrations (10 and 100 μmol/L) increased total K^+_e accumulation by 23% and 40%, respectively.

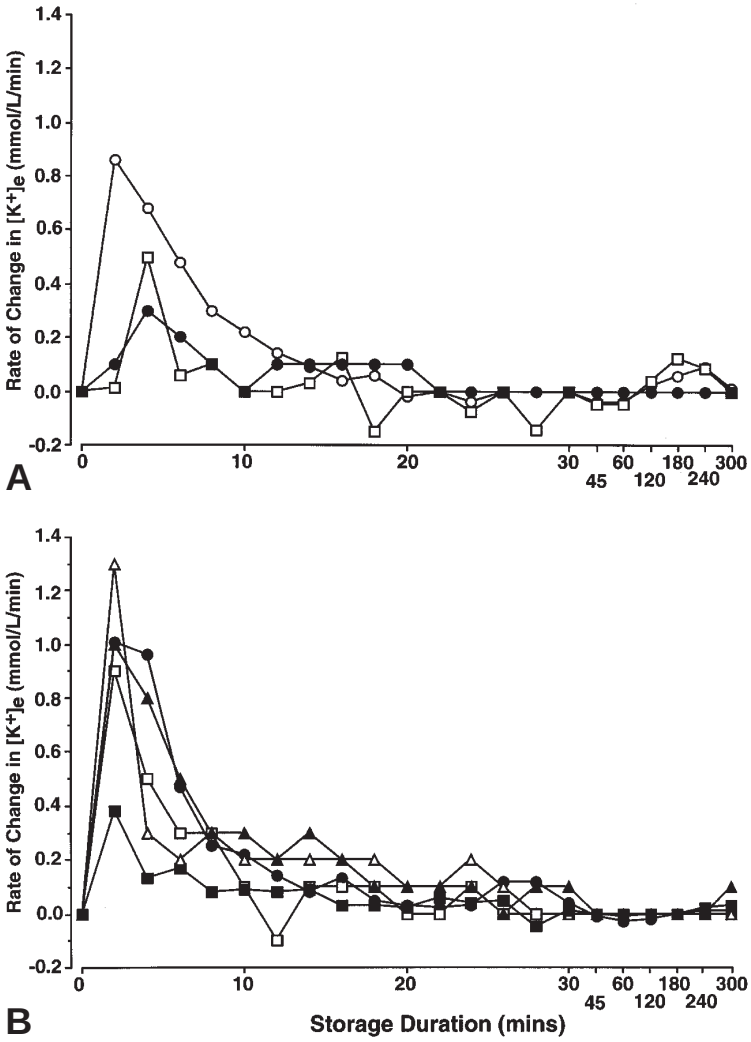

Fig 4. A, Rate of change in myocardial K^+_e during 5 hours of hypothermic (7.5°C) ischemic storage in hearts arrested and stored with Krebs-Henseleit buffer alone (control; *open circles*), high K^+ *(open squares)*, or tetrodotoxin *(filled circles).* Values represent the mean of 6 hearts per group; standard errors have been omitted for clarity. Note the change in scale along the time axis between 0-60 and 60-300 minutes. **B,** Rate of change in myocardial K^+_e during 5 hours of hypothermic (7.5°C) ischemic storage of hearts arrested and stored with Krebs-Henseleit buffer + 22 μmol/L tetrodotoxin + 0.1% methanol (control; *filled circles)* or control + 0.1 μmol/L *(open squares)*, 1.0 μmol/L *(filled squares)*, 10 μmol/L *(open triangles)*, and 100 μmol/L *(filled triangles)* furosemide. Values represent the mean of n = 4 hearts per group; standard errors have been omitted for clarity. Note the change in scale along the time axis between 0-60 and 60-300 minutes.

Effect of furosemide on rate of K^+_e accumulation during polarized arrest and storage. The rate of K^+_e accumulation during the first 30 minutes of storage in the control group and the 0.1, 1.0, 10, and 100 μmol/L furosemide-treated groups are shown in Fig 4, *B*. The peak rate of K^+_e accumulation was 1.01 ± 0.4, 0.9 ± 0.2, 0.4 ± 0.2, 1.3 ± 0.4, and 0.7 ± 0.3 mmol/L per minute, respectively, and occurred during the first 2 minutes of ischemia.

Myocardial water content. There were no differences in tissue water content between control (83.7% ± 0.7%) and 0.1 (82.9% ± 0.7%), 1.0 (83.4% ± 0.7%), 10 (81.8% ± 0.7%), and 100 μmol/L (83.6% ± 0.2%) furosemide-treated hearts after 5 hours of storage.

Effect of furosemide on recovery of function after polarized arrest and storage. Preischemic cardiac function did not differ before and after electrode insertion; thus preischemic cardiac function measurements were

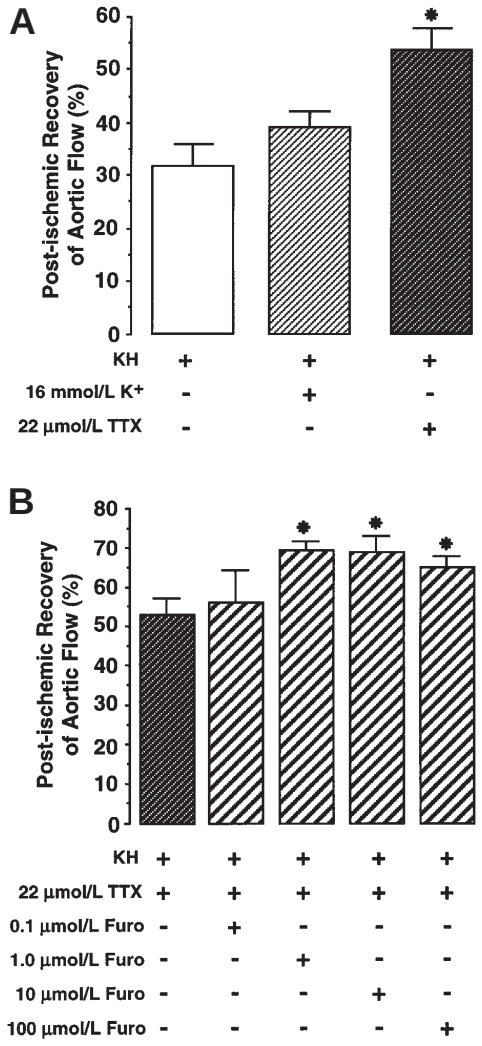

Fig 5. A, Postischemic recovery of aortic flow after 30 minutes of working mode reperfusion after K^+_e determination during 5 hours of hypothermic (7.5°C) ischemic arrest and storage with either Krebs-Henseleit buffer alone (control) or high K^+ and tetrodotoxin. Values are expressed as a percent of preischemic control value. Columns represent mean ± standard error of mean. **P* < .05 versus control, n = 6 hearts per group. **B,** Postischemic recovery of aortic flow after 30 minutes of working mode reperfusion after 5 hours of hypothermic (7.5°C) storage of hearts arrested and stored with 22 µmol/L tetrodotoxin + 0.1% methanol (control) or control + 0.1, 1.0, 10, or 100 µmol/L furosemide. Columns represent mean ± standard error of mean; **P* < .05 versus control, n = 8 hearts per group.

averaged (Table IV). Fig 5, *B*, shows the postischemic recovery of aortic flow after 5 hours of ischemic (7.5°C) storage. Recovery of aortic flow in the vehicle control and the 0.1, 1.0, 10, and 100 µmol/L furosemide-treated groups was 53% ± 4%, 56% ± 8%, 70% ± 2% (*P* = .04 vs control), 69% ± 4% (*P* = .04 vs control), and 65% ± 3% *(P* = .04 vs control), respectively. Recovery of other functional parameters (aortic pressure, heart rate, coronary flow, cardiac output, stroke volume, and stroke work) for all groups is shown in Table IV. Similar recovery profiles were observed with cardiac output, stroke volume, and stroke work. Heart rate, coronary flow, and aortic pressure recovered to similar values in all groups.

Table III. *Preischemic function and postischemic recovery (%) of cardiac function in hearts arrested and stored with ischemia (control), 16 mmol/L K^+ or 22 µmol/L TTX*

	Group		
	KH (control)	*KH + 16 mmol/L K^+*	*KH + 22 µmol/L TTX*
Heart rate			
beats/min	293 ± 7	281 ± 9	303 ± 20
% recovery	94 ± 2	89 ± 1	97 ± 3
Coronary flow			
mL/min	17 ± 1	18 ± 1	17 ± 1
% recovery	75 ± 4	83 ± 6	74 ± 4
Aortic flow			
mL/min	59 ± 1	61 ± 2	60 ± 1
% recovery	32 ± 4	39 ± 5	54 ± 4*†
Cardiac output			
mL/min	76 ± 2	79 ± 3	76 ± 1
% recovery	42 ± 4	48 ± 5	60 ± 4*
Aortic pressure			
cm H_2O	177 ± 5	183 ± 4	175 ± 2
% recovery	82 ± 1	82 ± 1	85 ± 1
Stroke volume			
mL/beat	0.26 ± 0.01	0.28 ± 0.01	0.26 ± 0.02
% recovery	44 ± 5	53 ± 5	60 ± 3
Stroke work			
$dyne^5$ cm/beat	46 ± 2	52 ± 2	46 ± 3
% recovery	37 ± 4	42 ± 5	51 ± 3

KH, Krebs-Henseleit; *TTX*, tetrodotoxin.
**P* = .02 versus control group.
†*P* < .05 versus 16 mmol/L K^+ group.

Discussion

Ischemia and K^+_e. Many studies have shown that K^+_e increases rapidly within seconds of the onset of ischemia and that there is a "typical" triphasic profile of K^+_e accumulation. After an initial rapid rise (phase 1), there is an intermediate phase (phase 2) in which K^+_e either plateaus[14] or declines,[15] and this is followed by a secondary increase in K^+_e (phase 3). In our control group of hearts (study A), the K^+_e profile was characteristically triphasic with a decline during the interme-

Table IV. *Preischemic function and postischemic recovery (%) of cardiac function in hearts arrested and stored with TTX in combination with various concentrations of furosemide*

	Group				
	KH + 22 μmol/L TTX + 0.1% methanol (control)	*KH + 22 μmol/L TTX + 0.1 μmol/L furosemide*	*KH + 22 μmol/L TTX + 1.0 μmol/L furosemide*	*KH + 22 μmol/L TTX + 10 μmol/L furosemide*	*KH + 22 μmol/L TTX + 100 μmol/L furosemide*
Heart rate					
beats/min	296 ± 6	297 ± 8	295 ± 5	280 ± 8	314 ± 12
% recovery	99 ± 4	95 ± 4	100 ± 3	98 ± 3	94 ± 3
Coronary flow					
mL/min	20 ± 1	19 ± 1	19 ± 1	18 ± 1	21 ± 1
% recovery	83 ± 8	76 ± 6	90 ± 4	84 ± 4	85 ± 6
Aortic flow					
mL/min	64 ± 2	63 ± 2	63 ± 2	61 ± 2	65 ± 2
% recovery	53 ± 4	56 ± 8	70 ± 2*	69 ± 4*	65 ± 3*
Cardiac output					
mL/min	84 ± 2	82 ± 3	82 ± 2	79 ± 3	85 ± 1
% recovery	60 ± 4	61 ± 8	74 ± 2*	72 ± 4*	70 ± 2*
Aortic pressure					
cm H_2O	193 ± 5	188 ± 3	191 ± 7	190 ± 7	190 ± 4
% recovery	82 ± 3	82 ± 3	86 ± 2	88 ± 1	87 ± 3
Stroke volume					
mL/beat	0.3 ± 0.004	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
% recovery	61 ± 5	64 ± 7	75 ± 3*	74 ± 3*	76 ± 5*
Stroke work					
$dyne^5$ cm/beat	55 ± 2	52 ± 2	54 ± 3	53 ± 3	52 ± 5
% recovery	51 ± 6	53 ± 7	65 ± 4*	68 ± 5*	67 ± 6*

KH, Krebs-Henseleit; *TTX,* tetrodotoxin.
*P = .04 versus control group.

diate phase of the profile. During phases 1 and 3 of the profile, K^+_e increased to 11 and 20 mmol/L, respectively, with the onset of phase 3 occurring approximately 90 minutes into the storage period. To our knowledge, this is the first time that measurement of K^+_e during hypothermic long-term storage has been reported.

During phase 1 (initial 30 minutes of ischemia) in hearts arrested with high K^+, the increase of K^+_e above baseline was lower than in the control group of hearts, as shown in Fig 3, *A.* This observation is consistent with the hypothesis that infusion of hyperkalemic solutions, which increase the K^+_e concentration, reduces the electrochemical gradient and the outward potassium driving force generated by the gradient. Our observations agree with those of Weiss and Shine,[14] who demonstrated that infusion of a hyperkalemic solution containing 16 mmol/L K^+ into rabbit myocardium also significantly attenuated K^+_e accumulation during phase 1 (initial 10 minutes) of *normothermic* global ischemia. In addition, hearts arrested with tetrodotoxin also exhibited a reduced level and rate of K^+_e accumulation during phase 1 compared with the control group of hearts. Whether this observation suggests that the sodium channel is directly or indirectly involved in the accumulation of K^+_e during early ischemia is unclear. During this initial period of ischemia, the opening of adenosine triphosphate–sensitive potassium channels (K^+ATP channels) has been implicated as contributing to the accumulation of K^+_e.[16] We[3] have shown in a previous study that levels of ATP and creatine phosphate are better preserved during the storage period in hearts arrested with tetrodotoxin than with high K^+. Higher ATP levels during ischemia in tetrodotoxin-arrested hearts may attenuate the opening of K^+ATP channels during early ischemia and reduce their contribution to overall K^+_e accumulation.

K^+_e accumulation during the late stages of unprotected ischemia have been correlated to the onset of irreversible injury and cellular uncoupling.[17] In the present study, K^+_e accumulation during the late phase in tetrodotoxin-treated hearts was lower than in both high K^+–treated and control groups. The time of onset of the second increase in K^+_e accumulation was also delayed, indirectly suggesting that tetrodotoxin-induced polarized arrest may be delaying the onset of irreversible injury and cellular uncoupling, which may account for

the improved recovery of function observed in these hearts.

K^+_e and Na^+ channel blockers. Mitani and Shattock[15] were able to demonstrate that R56865, a mixed blocker of the sodium-dependent potassium current (I_{KNa})[18] and cardiac sodium channel,[19] could reduce K^+_e accumulation during normothermic global ischemia. I_{KNa} reportedly becomes activated by concentrations of intracellular sodium (Na^+_i) in excess of 20 mmol/L.[20] In this present study, tetrodotoxin was clearly shown to reduce K^+_e accumulation compared with high K^+–treated and control unprotected hearts.

Until recently, the effects of selective sodium channel blockers on K^+_e accumulation during ischemia had not been studied. Shivkumar and colleagues[21] demonstrated, in the isolated perfused rabbit septum, that 20 μmol/L tetrodotoxin, 1 mmol/L furosemide, 5 μmol/L ethyl-isopropyl amiloride (EIPA), and 10 μmol/L verapamil reduced K^+_e accumulation during 30 minutes of normothermic hypoxia. They suggested that for "preservation of both electroneutrality and osmotic balance," net Na^+_i accumulation should reflect net K^+_e accumulation and that any interventions that reduce Na^+_i accumulation should also reduce K^+_e accumulation, thereby constituting a novel central role in the pathogenesis of ischemia and reperfusion. However, they did not assess postischemic function after treatment with tetrodotoxin, furosemide, EIPA, and verapamil, which would have strengthened their argument. Our observations suggest that cardioplegic arrest with tetrodotoxin can limit K^+_e accumulation during ischemia, which strongly supports Shivkumar's hypothesis that Na^+_i accumulation may play an integral role in K^+_e accumulation in the ischemic heart. Tetrodotoxin has been shown (1) to inhibit the I_{Naw} in sheep Purkinje fibers[2] and human ventricular myocytes[22] and (2) to attenuate Na^+_i accumulation induced by the sodium channel modulators, veratridine and lysophosphatidylcholine.[23] It is possible, therefore, that Na^+_i accumulation may have been attenuated by tetrodotoxin and subsequently delayed/attenuated I_{KNa} activation, which may partly explain the overall reduced K^+_e accumulation compared with high K^+–treated and control hearts. Sodium influx during ischemia, via the I_{Naw}, has previously been suggested to be involved in the pathogenesis of ischemia/reperfusion injury.[24]

Dual effects of furosemide on K^+_e accumulation during polarized arrest and storage. In this study, K^+_e accumulation during hypothermic (7.5°C) storage was reduced by 0.1 and 1.0 μmol/L furosemide; these observations are consistent with data from Mitani and Shattock,[15] in which 100 μmol/L furosemide was shown to reduce K^+_e accumulation during normothermic (37°C) ischemia. They also demonstrated a slight augmentation of K^+_e accumulation during the late phase of their ischemic duration with a higher concentration of furosemide (1.0 μmol/L). This is again consistent with our results in which we observed elevated K^+_e accumulation at furosemide concentrations of 10 and 100 μmol/L, although we observed this elevation throughout the K^+_e profile during hypothermic ischemia. Thus furosemide appears to exert a dose-dependent dual effect on myocardial K^+_e accumulation.

During steady state conditions, a significant proportion of K^+ is thought to enter the cell via $Na^+/K^+/2Cl^-$ cotransport,[25] although the majority of K^+ will enter via the sodium pump. Inhibition of the sodium pump by profound hypothermia[28] (as in the present study) may increase the proportion of K^+ entry via the $Na^+/K^+/2Cl^-$ cotransporter. This would tend to have the added effect of increasing Na^+_i. Thus maximal inhibition of the $Na^+/K^+/2Cl^-$ cotransporter by high concentrations of furosemide may result in a net increase in K^+_e accumulation but should also attenuate the increase in Na^+. This may also explain our paradoxic observation of elevated K^+_e together with significant improvement in myocardial function at the higher concentrations of furosemide.

At relatively low furosemide concentrations (which are close to the 50% inhibitory concentration of 5 μmol/L),[26] only partial inhibition (up to 50%) of the cotransporter would occur. It might be expected, therefore, that lower concentrations of furosemide elevate K^+_e accumulation to a lesser extent than the higher doses of furosemide; in fact, K^+_e accumulation was reduced to below control levels. Inasmuch as the flux of both Na^+ and K^+ via this mechanism is unidirectional,[12] it is tempting to speculate that an alternative Na^+- and K^+-coupled mechanism is involved in mediating this dual effect. One possible candidate is the sodium-dependent potassium current (I_{KNa}).[20] Therefore, even though low concentrations of furosemide (lower than the published 50% inhibitory concentration) may mediate a small elevation of K^+_e because of reduced K^+ influx by $Na^+/K^+/2Cl^-$ cotransport inhibition, the net K^+_e accumulation is attenuated because of a possible reduction or delay in the activation of I_{KNa} and its contribution to K^+_e accumulation. Interestingly, the concentrations of furosemide that attenuated K^+_e accumulation in this study have also been shown[10] to significantly attenuate Na^+_i accumulation during hypothermic storage, thereby supporting our contention that improved function is related to reducing Na^+_i accumulation.

Functional recovery and possible mechanisms of protection. Exposure of myocardial tissue to a hyperkalemic (16 mmol/L K^+) solution has been shown to increase intracellular calcium (Ca^{2+}_i).[6] As a conse-

quence, energy-consuming mechanisms that maintain calcium homeostasis may exert a cellular metabolic demand and accelerate the depletion of energy stores.[27] Sternbergh and colleagues[8] reported that hearts perfused with a solution containing 25 μmol/L tetrodotoxin demonstrated a significantly lower oxygen consumption than hearts perfused with a high K^+ solution. We[3] have previously demonstrated that hearts arrested with tetrodotoxin had a significantly higher level of ATP and creatine phosphate at the end of 5 hours of hypothermic storage.

The consequences of Na^+_i accumulation become apparent during reperfusion. The ability of the sodium pump to efficiently remove accumulated Na^+_i during early reperfusion may be attenuated because of reduced levels of high-energy phosphates[28] or the presence of lipid metabolites.[29] As a consequence, Na^+_i is thought to be extruded via reverse mode Na^+/Ca^{2+} exchange, resulting in a subsequent calcium overload and poor recovery of function. Tani and Neely[11] have shown that the extent of calcium overload during reperfusion is directly proportional to the degree of Na^+_i accumulation during ischemia. In our study, we speculate that tetrodotoxin-induced polarized arrest reduced Na^+_i and Ca^{2+}_i accumulation in comparison with high K^+ depolarized arrest and that further reduction of Na^+_i accumulation by furosemide-mediated inhibition of $Na^+/K^+/2Cl^-$ cotransport during hypothermic polarized arrest may have led to an additive reduction of calcium overload on reperfusion and consequent improvement in the recovery of function. This is supported by our measurements of reduced ionic movement in the present study, represented by the reduced K^+_e accumulation in the tetrodotoxin group (study A). This reduction in ionic imbalance correlates with improved recovery of function.

In the present study, we have demonstrated that furosemide exerts dose-dependent beneficial effects on recovery of function in tetrodotoxin-induced polarized long-term myocardial preservation of the rat heart. We believe this is due to inhibition of the $Na^+/K^+/2Cl^-$ cotransport system, which reduces Na^+_i accumulation. Rubin and Navon[10] have shown, using ^{23}Na nuclear magnetic resonance spectroscopy, that both furosemide and bumetanide can significantly attenuate Na^+_i accumulation.

In addition, the dose-response effects of furosemide showed a plateau and a slight reduction in the recovery of postischemic function; at the highest concentrations of furosemide investigated, this plateau and trend toward reduced function was accompanied by higher K^+_e accumulation in comparison with the hearts stored with 0.1 and 1.0 μmol/L furosemide. Rubin and colleagues[30] reported a bell-shaped relationship between the concentration of furosemide and the recovery of cardiac function after cardioplegic arrest; at a furosemide concentration of 1.0 mmol/L, hearts failed to exhibit any recovery of cardiac function. The elevated K^+_e accumulation observed in our studies may, in part, explain the apparent toxic effects of higher concentrations of furosemide observed by Rubin and associates.[30]

Limitations of the studies. One of the main limitations of this study relates to the fact that the hearts were perfused with crystalloid buffer when blood perfusion would have been more physiologic. However, a blood-perfused preparation would have been more difficult than a crystalloid-perfused preparation, particularly for a working heart preparation. We used the working heart preparation specifically because we wished to examine the ability of the heart to pump against an afterload after a prolonged preservation period. Most blood-perfused rat heart preparations use Langendorff perfusion, which does not allow pump function to be measured. Another confounding factor relating to blood perfusion for this particular study could be the potential hemolysis and the associated increase in extracellular K^+, which would influence our measurements of this parameter. Crystalloid perfusion, and the associated low oncotic pressure that causes myocardial edema, may also have influenced our inability to detect any furosemide-induced changes in tissue water content.

Another limitation relates to our inability to directly examine the mechanism by which we propose that polarized arrest and inhibition of $Na^+/K^+/2Cl^-$ cotransport influences cardioprotection, namely the attenuation of intracellular Na^+ accumulation. Measurement of Na^+_i during storage was beyond the scope of this study: however; it would be of considerable interest for future studies. We have indirectly demonstrated that ionic imbalance is important in long-term myocardial preservation by the measurement of K^+_e accumulation.

Although direct extrapolation of the results in the rat heart to clinical heart transplantation and human organ storage should be approached with considerable caution, we do consider that the concepts explored in this study should be applicable in the human heart. We do not advocate using tetrodotoxin for human heart preservation, but other sodium channel blockers that are in current clinical use (such as lidocaine) may be useful, and further studies using these compounds would be of considerable interest.

Conclusions

The observations of this study support our hypotheses (1) that tetrodotoxin-induced polarized arrest attenuates ionic changes (lower K^+_e accumulation) and improves the recovery of cardiac function when com-

pared with high K^+ depolarized arrest and storage and (2) that $Na^+/K^+/2Cl^-$ cotransport inhibition by furosemide at an optimal concentration of 1.0 μmol/L improves preservation conferred by polarized arrest by further limiting ionic imbalance and reducing K^+_e accumulation during hypothermic storage. However, high concentrations of furosemide may lead to an elevation of myocardial K^+_e accumulation and impair the recovery of cardiac function.

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